PROJECT 11-SP-11X Notebook No. [redacted]/81052

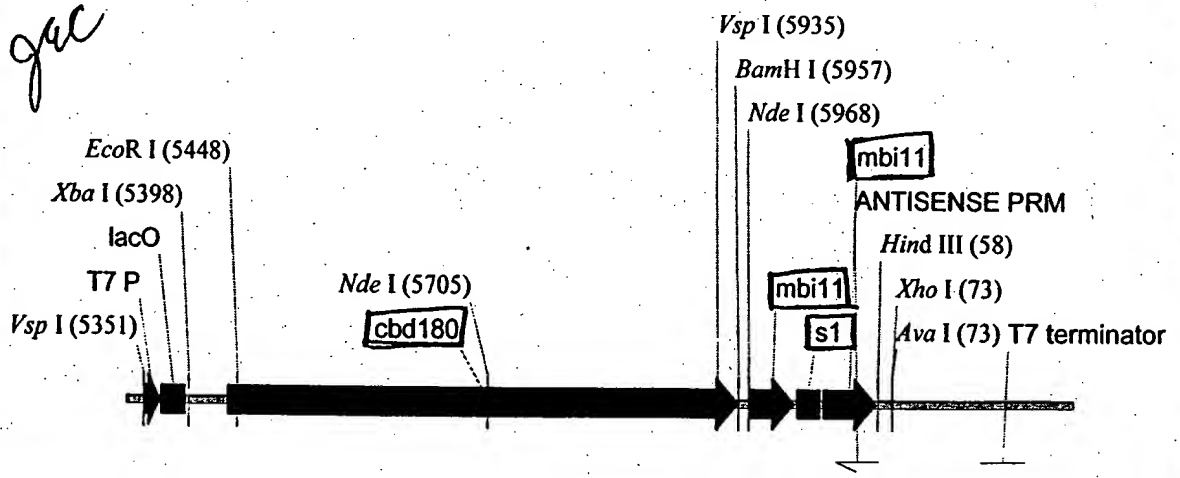
Continued From Page 1

TITLE: PREPARATION OF 2×MBI-11 CONTAINING A SPACER REGION

PURPOSE:

To insert a previously prepared cassette containing MBI11 (no stop codon) followed by a 8 amino acid (25 base pair) fragment, the spacer. This particular spacer is a natural sequence from Apis mellifera (honeybee); it occurs between identical copies of genes producing apidaecins. Hopefully the spacer will have a similar effect to its natural version - to allow expression of multiple tandem copies of toxic genes. The cassette will be inserted into pET21CBDX-11 - i.e., an already-expressing vector with a copy of MBIN that has a stop codon in at the end. The cassette will be inserted such that the MBI11 with the stop codon will remain the last copy in the tandem sequence.

The final product will be as follows:



Fragment of pET21CBDX-2X11s1
965 bp (molecule 6042 bp)

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Testing expression of poly mbl-11 clones in enriched media)

Strategy: Want to compare the expression of pET21CBDX-1X11 w/ the other mutagenic mbl-11 clones, as it seems that expression decreases w/ increasing number of mbl-11 codons. It seems plausible that by enriching the media of lysine & tryptophan that it may be possible to increase expression, especially of the multiple mbl-11 clones. (Since the amino acid sequence of mbl-11 is rich in lysine & tryptophan). Will compare expression by optimizing the amount that is loaded on the SDS-PAGE gels.

Methods:

25mg/mL L-lysine stock solution: 250mg L-lysine (lot #SLH121175) dissolved in 10mL sterile ddH_2O (in ddH_2O)
- filter sterilized thru 0.2μm filter

12.5mg/mL L-tryptophan stock solution: 250mg L-tryptophan (lot #BH0352Z5) in 19.8mL ddH_2O, 172μL conc. HCl. (in 0.1N HCl)
- filter sterilize thru 0.2μM filter

Enriched Terrific Broth - add 18μL of stock lysine soln & 36μL of stock tryptophan soln to 18mL TB.

Inoculate 3mL TB (w/ 100μg/mL amp + 30μg/mL kan) w/ pET21CBDX-1X11, or pET21CBDX-2X11, or pET21CBDX-3X11, or pET21CBDX-4X11. (all in XL-1 + pGP1-2).
Incubate @ 30°C, w/ shaking.
Also inoculate 2mL TB w/ 30μg/mL kanamycin w/ XL-1 + pGP1-2.

Prepare 4x 3mL enriched TB w/ 100μg/mL amp + 30μg/mL kan.
Inoculate w/ pET21CBDX-1X11 / 2X11 / 3X11 / 4X11 in XL-1 + pGP1-2.
Also inoculate 2mL enriched TB w/ 30μg/mL kan w/ XL-1 + pGP1-2.
Incubate @ 30°C, w/ shaking.

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Induce expression of 1mL overnight culture given @ 30°C by adding 1mL fresh TB (+ Amp) without lysine + tryptophan), shaking @ 42°C, 3 hours.

Test A_{600} of overnight cultures: (1/6x dilution)

Enriched media:
- XL-1 + pGP1-2 : 0.603
- pET21CBDX-1X11 : 0.445
- " -2X11 : 0.091
- " -3X11 : 0.339
- " -4X11 : 0.081

Normal media:
- XL-1 + pGP1-2 : 0.541
- pET21CBDX-1X11 : 0.476
- " -2X11 : 0.111
- " -3X11 : 0.345
- " -4X11 : 0.093

Test A_{600} of induced cultures: (1/6x dilution).

Normal media:
- ~~XL-1 + pGP1-2~~ pET21CBDX-1X11 : 0.430
- " -2X11 : 0.097
- " -3X11 : 0.231
- " -4X11 : 0.138

Enriched media:
- pET21CBDX-1X11 : 0.425
- " -2X11 : 0.139
- " -3X11 : 0.220
- " -4X11 : 0.103

* Spin down cells equivalent to OD = 1.5. Resuspend in 100uL SDS-Reducing buffer. Boil samples, 10'. Load 10uL = O.D = 0.15 of each sample onto 15% SDS-PAGE.

Note: pET21CBDX-4X11 - 42°C sample must have accumulated water during boiling step. Loaded 20uL (eyeball estimate) - prepare another sample for use next time.

* Also resuspended to equal OD = 1.5 (then resuspended in 100uL reducing buffer).

Overnight cultures: Enriched media):
- XL-1+pGP1-2 - 173uL
- pET21CBDX-1X11 - 197uL
- " 2X11 - 845uL
- " 3X11 - 272uL
- " 4X11 - 1.008mL

Enriched media):
- XL-1+pGP1-2 - 155uL
- 1X11 - 209uL
- 2X11 - 1.030mL
- 3X11 - 271uL
- 4X11 - 1.057mL

Induced cultures:
- pET21CBDX-1X11 - 218uL
- 2X11 - 966uL
- 3X11 - 406uL
- 4X11 - 679uL

Enriched media:
- 1X11 - 221uL
- 2X11 - 674uL
- 3X11 - 426uL
- 4X11 - 910uL

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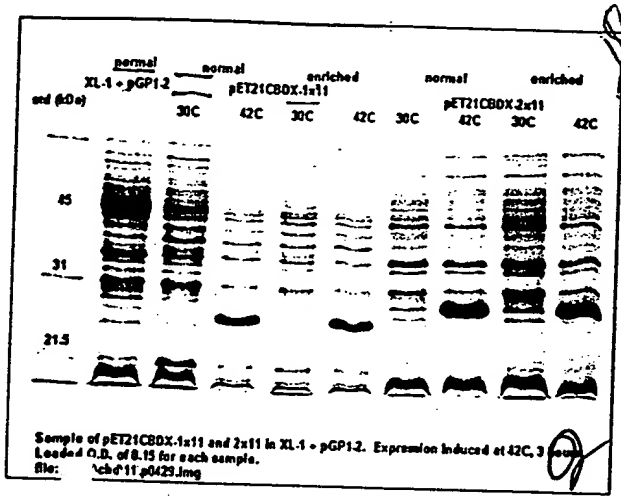
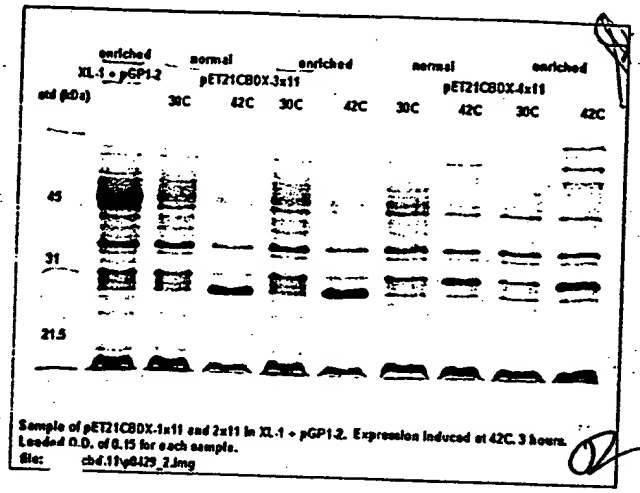
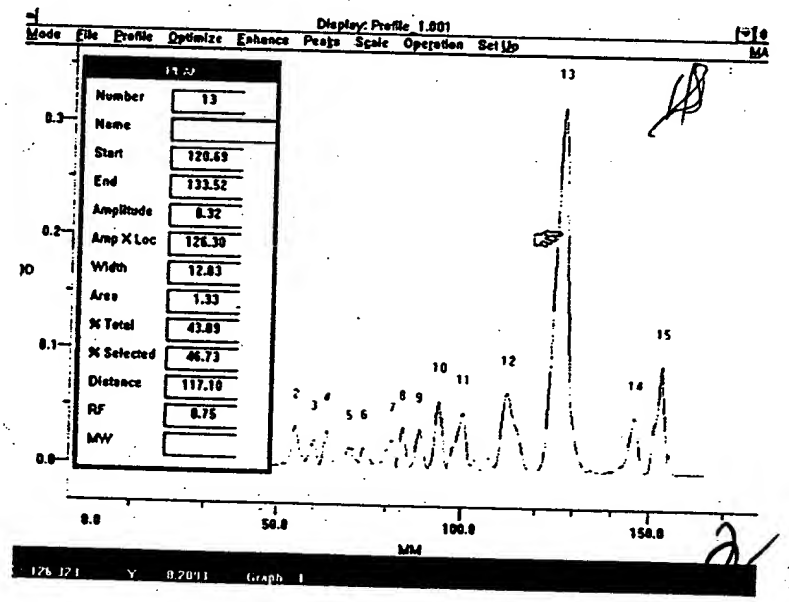




Good expression.
No profile analysis on protein gels.

pET21CBDX-1X11:



CBDX = 180 amino acids)
1X11 = 13 aa.

Serum protein = 43% of total protein

1X11 = 6.7% peptide in serum protein

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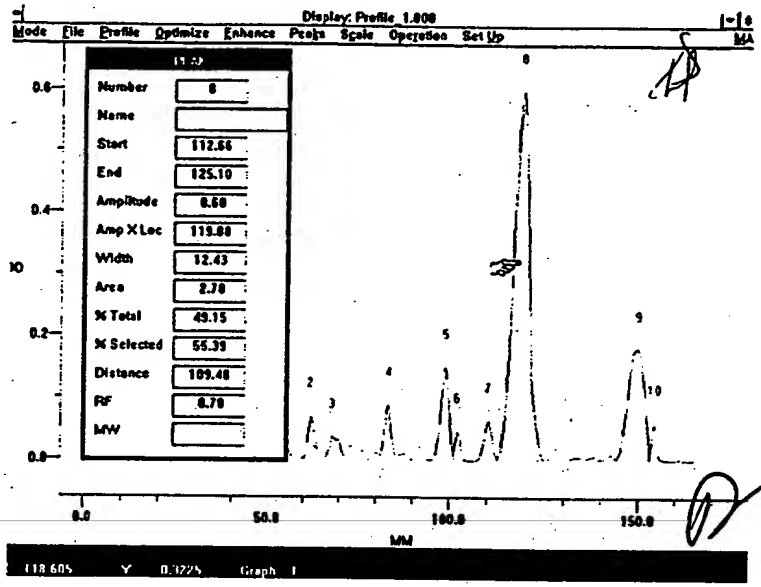
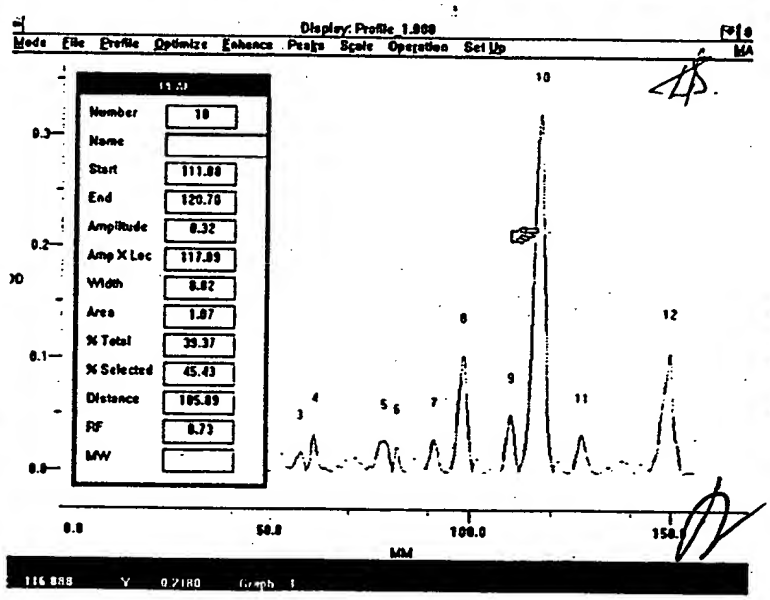
PET21CBDX-2X11



fusion protein = 49% of total protein

2X11 = 13.6% peptide in fusion protein

PET21CBDX-3X11



fusion protein = 39% of total protein

3X11 = 17.8% of fusion protein = peptide

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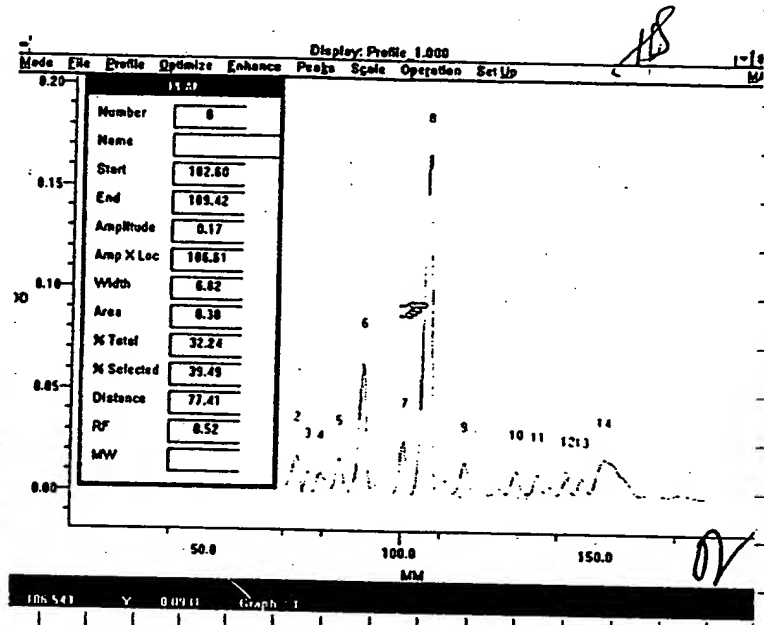
pET21CBDX-4X11



fusion protein = 32% of total protein

peptide = 22.4% of fusion protein

But same band was found @ 30°C (uninduced sample) → will redo expression of pET21CBDX-4X11.

Summary:

mbi-11 clone	% expression of fusion protein	% peptide	% peptide expression
1x11	43.09	6.735751295	2.902435233
2x11	49.15	12.62135922	6.203398058
3x11	39.37	17.80821918	7.01109589
4x11	32.24*	22.4137931	7.226206897*

*percentages are lower when the 30°C (uninduced) sampled are taken into consideration.

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TITLE: EXPRESSION OF CBDX-2x11S1 FUSION PROTEIN IN E.COLI MC4100(F) AND BL21(DE3)

PURPOSE:

Two strains were previously prepared:
1.) MC4100 (F) + pGP1-2 + pET21CBDX-2x11S1
2.) BL21(DE3) + pET21CBDX-2x11S1
(see pp. 92-100, MB1058, and pp. 1-22, MB1082).

Both of these strains should be capable of expressing the CBDX-2x11S1 fusion protein via the T7 promoter system (in MC4100(F), after induction at 42°C; in BL21, constitutively with overnight growth).

In this experiment I will test fusion protein expression from each of these strains using the appropriate induction methods.

EXPERIMENTAL DETAILS:

I GROWTH OF STRAINS

Overnight Growth

- four culture tubes were prepared: 2 containing 2 mL TB + 100 µg/mL Amp, and 2 containing 2 mL TB + 100 µg/mL Amp + 30 µg/mL Kan.
- the Amp tubes were both inoculated with the plasmid prep O/N culture (see p. 22) of sample #2 (BL21)
- the Amp + Kan tubes were both inoculated with the plasmid prep O/N culture (see p. 22) of sample #11 (MC4100)(F)
- tubes were placed @ 37°C / 30°C O/N with shaking

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- note: these O/N cultures from plasmid prep are quite old (several days), so I'm preparing 2 cultures of each and will test for presence of plasmids following O/N growth.

HARVESTING OF BL21 SAMPLES

Harvesting

- removed 100 µl from BL21 O/N's → @ 13,200 rpm 1 min → removed S/N
- resuspended pellet in 100 µl SDS buffer
- froze @ -20°C

INDUCTION OF MC4100 (F) SAMPLES

Removal of Preinduction Samples

- removed 100 µl from MC4100 (F) O/N's and harvested as described above → these are the preinduced samples
- resuspended in 100 µl & froze @ -20°C.

Induction

- removed 0.9 ml of each MC4100 (F) O/N into a fresh sterile culture tube
- added 0.9 ml TB to each
- placed @ 42°C for 3 h.

- following induction, removed 200 µl → harvested as described above
- resuspended in 100 µl → froze @ -20°C.

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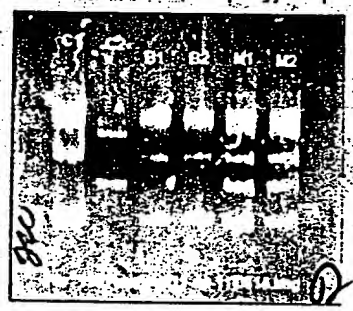
IV PLASMID PREPS

Plasmid Preps

- during induction time, removed remainder of all 4 O/N cultures to microcentrifuge tubes (~1 mL) and performed plasmid preps as per SOP.
- stopped after NH_4OAc precipitation
- resuspended in 50 µL TE + RNase.

Gel

- ran 1 µL of each prep with 1 µL pGP1-2 + pET21CBDX-2x11S1
- ran on 1% agarose for 45' @ 80V.



C1 = pET21CBDX-2x11S1
C2 = pGP1-2.

- appropriate plasmids are all there!
- need only test expression of one of each pair.

V TESTING OF EXPRESSION LEVEL

Preparation of Samples

- boiled samples for 10 min.
- spun down

Gel

- prepared ~~@~~ 4% a 15%/4% stacking gel
- loaded 10 µL of BL21 (1) and MC4100(K) (1), along with an expressing 2x sample from Jan

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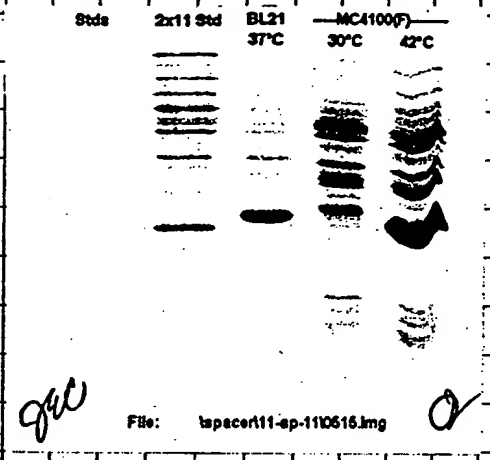
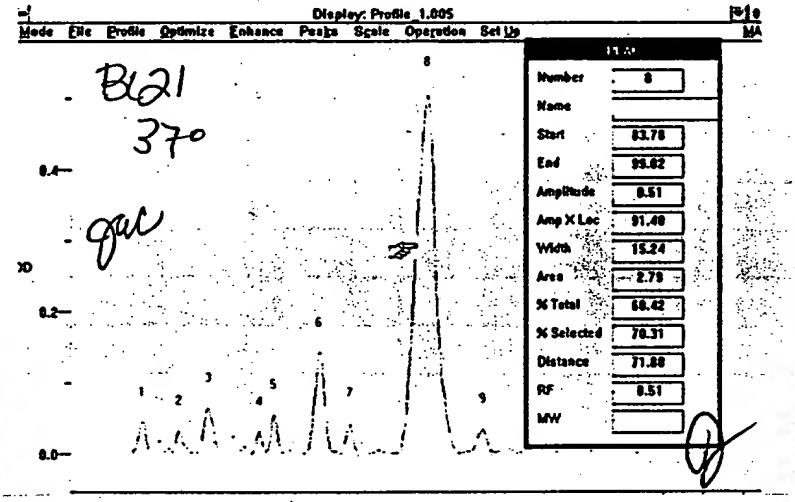
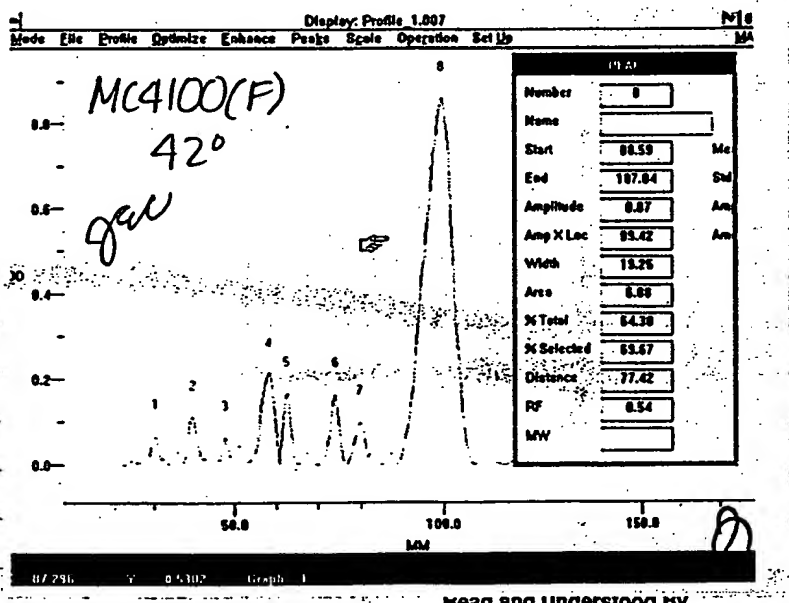
ran @ 100V for ~100 min



qw

Profile Analysis:





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- apparently very high expression!
- however, band appears too big - maybe a frame-shift mutation somehow?
- will do sequencing to check.

- NOTE: insert also looked too big - see p. 7, fig. 9.

DISCUSSION:

Expression of 2x11S1 fusion protein, at apparently high levels, was achieved in both BL21(DE3) and MC4100(F). However, the fusion protein band appears perhaps slightly larger than expected. The clone will be sequenced.

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TITLE: TESTING THE EXPRESSION OF CBD96-4x11 AND CBD96-2x11B7 FUSION PROTEIN IN E.COLI MC4100(F)

PURPOSE:

In a previous experiment (see pages 35-44, this book) the expression of CBD96-6x11 and -10x11 fusion protein was tested and determined to be nil. In this experiment, CBD96-4x11 will be tested for expression to see whether it expresses at a greater level than CBD-4x11.

In addition, Jan has prepared a CBD96-2x11B7 clone. This clone was prepared from a 2x11B7 fragment that was prepared in one piece - i.e., it wasn't constructed from two 1x clones. MB11B7 is, as of now, our "official lead compound" so expression at high levels is essential. The expression of this fusion protein will also be tested.

EXPERIMENTAL DETAILS:

I CREATION OF TEST STRAINS

Electroporation

- used MC4100 (F) as the host strain
- chose clones #8 + 12 of 2x11B7, and #4-4 and 4-6 of 4x11
- electroporated plasmid DNA, from Jan Burian 1 µl 2x11B7 clones or 2 µl 4x11 clones (low cDNA) with 1 µl pGP1-2.
- plated 10 µl and 50 µl on Mac + Amp + Kan
- grew O/N at 30°C.

- few colonies - especially for 4x11 clones
- but at least one colony on each 50 µl plate

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II OVERNIGHT GROWTH OF STRAINS

Growth

- prepared four culture tubes containing 3 mL TB + 100 µg/mL ampicillin + 30 µg/mL kanamycin
- inoculated each tube from with a single colony from one of the 50 µL electroporation plates
- grew O/N at 30°C with shaking (300 rpm).

III PREINDUCTION HARVESTING & INDUCTION OF EXPRESSION

Preinduction Harvesting

- forgot to remove preinduction samples!
- so added 100 µL fresh TB to tubes after they were empty and re-grew at 30°C for ~3h.
- then harvested (100 µL) → ¢ → removed S/N
- resuspended in 100 µL SDS reducing buffer.

Induction of Expression

- removed 1 mL of each sample and put in a tube containing fresh TB
- incubated @ 42°C with shaking (300 rpm) for 3h.

IV CHECKING FOR PRESENCE OF PLASMID

Plasmid Preps

- meanwhile, while samples are expressing, use rest of O/N cultures (2 mL) to do plasmid preps
- did as per SOP to NH_4OAc precipitation
- resuspended pellets in 50 µL TE + RNase

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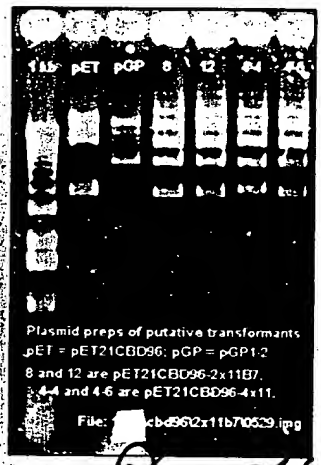
Gel

- prepared a 1% agarose gel
- loaded 3 µl pET21CBD96 (from QIA) and 3 µl pGP1-2 as controls
- loaded 1 µl each plasmid prep and 5 µl 1 Kb ladder.
- ran 45' @ 80V.

Figure 1 Agarose gel of putative transformants. All clones are positive for the appropriate plasmids.



V CHECKING FUSION PROTEIN EXPRESSION

Harvesting of Expressed Plasmid Samples

- removed samples from 42°C incubation after 3h.
- extracted 200 µl per tube → ↓ → removed SN
- resuspended each pellet in 100 µl SDS buffer.

Preparation of Samples

- removed all samples (30°C and 42°C) from storage at -20°C.

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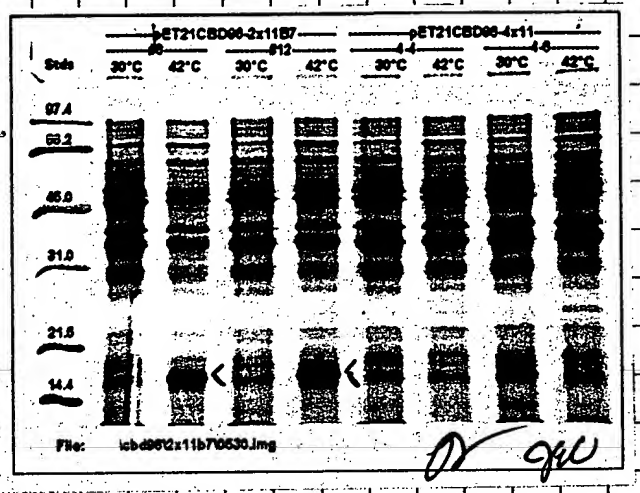
- boiled samples for 10 min, then spun down.

Gel

- prepared a 15%/4% stacking PA gel
- loaded 10 µl of each sample with 5µl low molecular weight protein standards.
- ran 45' @ 200V.

Figure 2 Expression of 2x11B7 and 4x11 from CBD96.



- since 2x11B7 is being expressed at fairly low levels and 4x11 isn't expressed at all, I'll check to see whether any/all of the fusion protein is soluble in the cell as opposed to sequestered in inclusion bodies.

VI OVERNIGHT GROWTH

Growth

- set up four new culture tubes containing 25 TB + 100 µg/ml ampicillin + 30 µg/ml kanamycin
- inoculated using loopfuls of last night's overnights (30°C).

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- grew o/n at 30°C, with shaking (300 rpm)
- NOTE: in morning, incubator was at ≥ 32°C and "Hi-Temp" alarm was flashing.

VI PREINDUCTION HARVESTING & INDUCTION OF EXPRESSION

Preinduction Harvesting

- in morning, removed ~~20~~ 100 µl of each culture to an Eppindorf tube → ↓ → removed S/N.
- resuspended pellet in 100 µl SDS reducing buffer
- froze @ -20°C until needed

Induction of Expression

- prepared 4 fresh culture tubes, each containing 0.9 mL TB
- added 0.9 mL culture to tubes
- incubated @ 42°C for 3h with shaking (300 rpm)

VII CHECKING FUSION PROTEIN EXPRESSION - SOLUBLE/INSOLUBLE

Harvesting of Postinduction Sample

- removed 200 µl of each sample after incubation
- ↓ → removed S/N → resuspended in 100 µl SDS reducing buffer

Sonication to Release Soluble Cell Components

- transferred 1 mL induction culture to an Eppendorf → ↓ and removed S/N
- resuspended in 980 µL H_2O + 20 µl 0.5 M EDTA (final [EDTA] = 0.010 M) → vortexed to mix
- Sonicated @ 10 (amplitude) using microtip: 1 minute × 3 times (kept on ice between)

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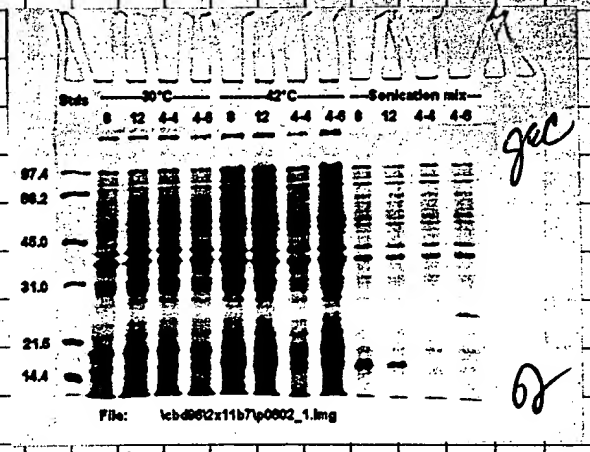
Harvesting of Sonicated Samples

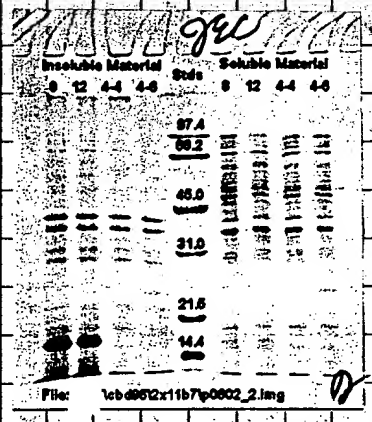
- removed 100 µl each sample → diluted w/ 100 µl SDS reducing buffer
  → this is the SONICATED sample
- removed remaining sample
  - resuspended pellet in 400 µl buffer
    → this is the INSOLUBLE sample
  - transferred 200 µl S/N to separate tube and added 200 µl buffer
    → this is the SOLUBLE sample

Gels

- prepared two 15%/4% stacking PA gels.

Figure 3 PA gels of expression samples. 10 µl per well.





- prepared an additional 15%/4% gel and ran the 4-6 samples only, trying to "even out" loading to get ~equal amount of material per lane

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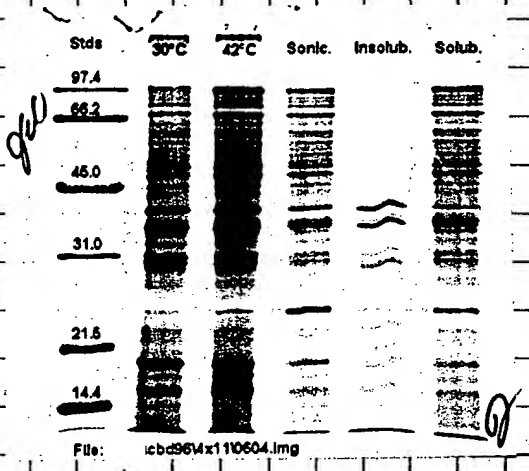
- i.e., loaded 7.5 µl 30°C
5 µl 42°C
12.5 µl sonic.
12.5 µl insol.
12.5 µl sol.
- ran @ 200V, 45'.

Figure 4 Expression samples of pET21/CBD96-4x11 clone #4-6.



DISCUSSION:

Good expression of pET21/CBD96-2x11B7 was obtained. It appears from figure 3 that CBD96-2x11B7 fusion protein is insoluble.

Interestingly, a low level of CBD96-4x11 fusion protein was expressed, but it appeared to be soluble, with only a very light band appearing in the "insoluble" lane. For unknown reasons, the fusion protein band is not visible in the 42°C lane. Perhaps this sample was not completely lysed prior to loading.

If CBD96 fusion proteins are soluble, this could cause many problems in later purification steps.

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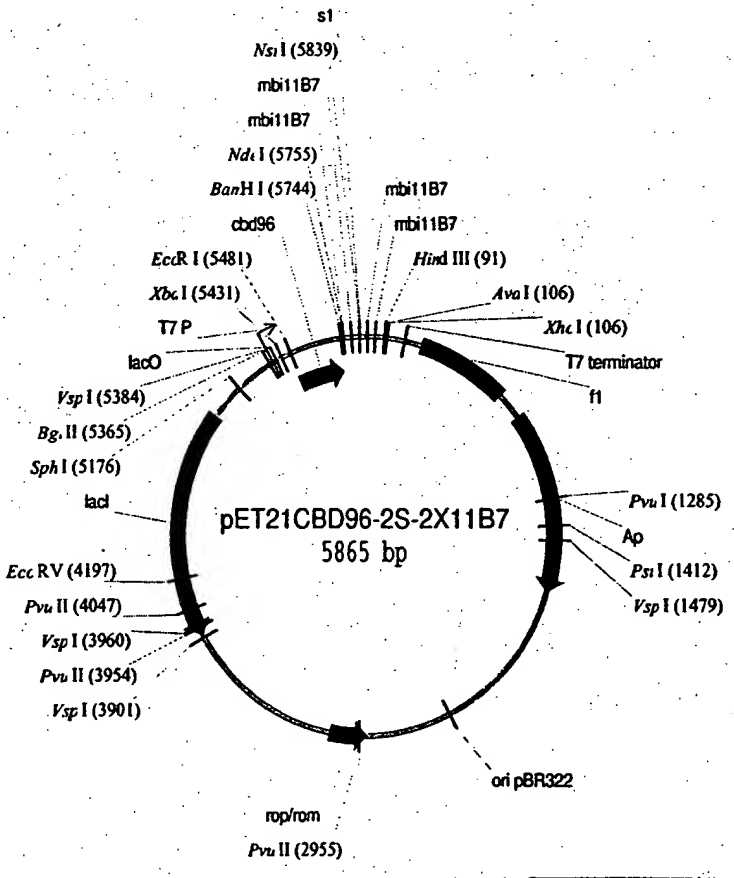
TITLE: TESTING EXPRESSION OF CBD96-11B7-sp-2x11B7 AND CBD96-2x11B7-Sp-2x11B7 FUSION PROTEINS

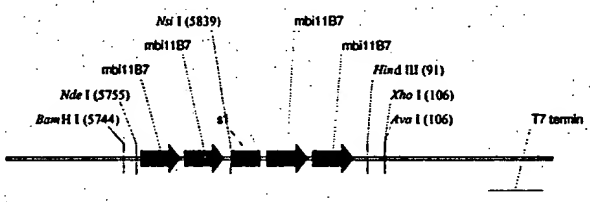
PURPOSE / BACKGROUND INFO:

I have obtained pET21CBD96-11B7-sp-2x11B7 and pET21CBD96-2x11B7-sp-2x11B7 cloned from Jan. These are as shown in figure 1.

Figure 1 3x11B7sp and 4x11B7sp clones used in this experiment.



From pET21CBD96-2x11B7(LAST) + pBLKS-V-2x11B7S



Fragment of pET21CBD96-2S-2X11B7
474 bp (molecule 5865 bp)

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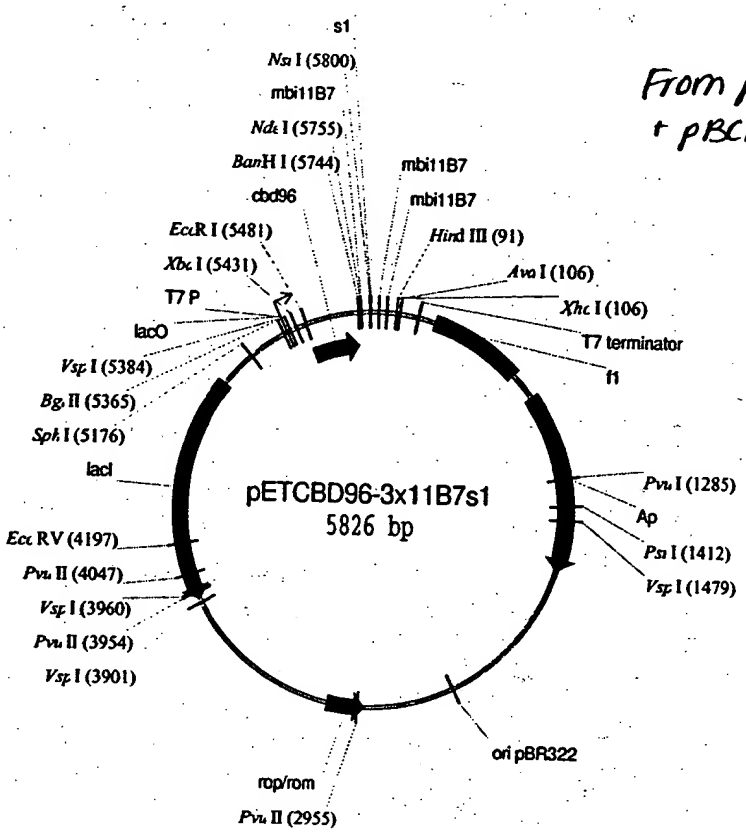
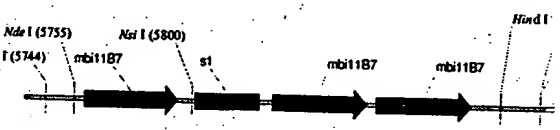


From pET21CBD96-2x11B7(LAST) + pBCKS-V-11B7S. JW



Fragment of pETCBD96-3x11B7s1
208 bp (molecule 5826 bp)

DJ

I will now create expression strains containing these clones and test for ability to express fusion protein.

EXPERIMENTAL DETAILS:

I CREATION OF EXPRESSION STRAINS

Electroporation

- obtained DNA from Jan

Figure 2 B/H digests of Jan's clones which showed inserts of correct size (i.e., all of these clones are positive).

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- selected 3X clones #1, 12 and 4X clones #2, 10 to work with further

- electroporated each of these into MC4100(F), using 1 µl plasmid DNA and 1 µl pGP1-2, as per SOP
- plated 10 µl of each → incubated 30°C O/N (Mac + Kan + Amp)
- very few colonies!
- only 2 plates (#10 & #12) have any colonies - and only a few colonies each!
- luckily, this includes a 3X and 4X plate so I can continue with the experiment

PREPARATION OF OVERNIGHT CULTURES

Overnights

- prepared two culture tubes, each with 3mL TB + 100 µg/mL Ampicillin + 30 µg/mL Kanamycin
- inoculated one tube with #10 and the other with #12 (using toothpicks)
- grew both tubes O/N @ 30°C, 300 rpm.

VERIFICATION OF PRESENCE OF PLASMIDS

Plasmid Preps

- removed 1.8 mL of each overnight culture to a fresh microfuge tube
- did plasmid preps as per SOP
- stopped after isopropanol ppt → resuspended in 100 µl TE + RNase

Gels

- prepared a 1% agarose gel

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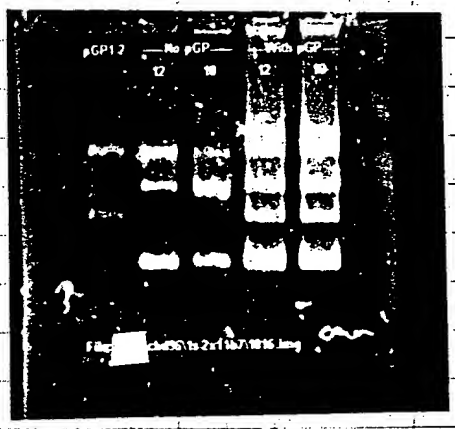
- loaded 2 µl each of: pGPI-2, #12, #10, #12 w/pGP, #10 w/pGP
- ran gel 60' @ 60V.

Figure 3 Plasmid preps of putative transformants. Both are positive for pGPI-2.



V INDUCTION OF EXPRESSION

Harvesting of Preinduction Cultures

- from each O/N culture, removed 100 µl to a a fresh microfuge tube
- @ 13,000 rpm, 1' → removed S/N
- resuspended each pellet in 100 µl SDS reducing buffer
- froze @ -20°C until needed.

Induction of Culture

- removed 1mL of each O/N culture to a fresh culture tube containing 1mL fresh TB
- incubated 3h @ 42°C, with shaking (300 rpm)

V CHECKING FOR FUSION PROTEIN EXPRESSION

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Harvesting of Induction Culture

- removed 200 µl of each culture to microfuge tubes
- @ 11 → removed SN
- resuspended each pellet in 100 µl SDS reducing buffer

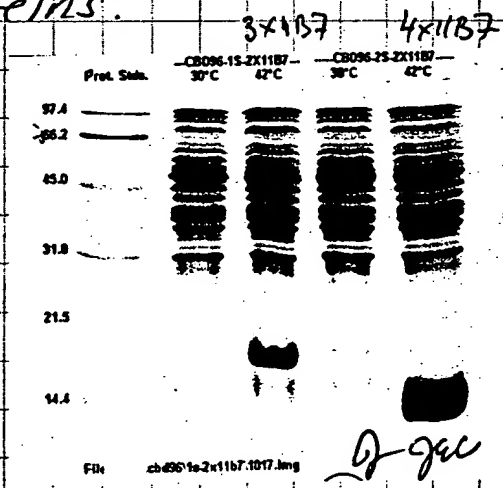
Preparation of Samples

- removed preinduced samples from freezer
- boiled all samples 10'
- spun down to concentrate

Gel

- prepared a 15% / 4% stacking gel
- loaded 10 µl each sample, along with 5 µl low MW standards.
- ran gel @ 100V for 2h then @ 200V for 10'

Figure 4 Expression of CBD96-1S-3x11B7 and CBD96-2S-4x11B7 fusion proteins.



- expression of 1S-2x11B7 is excellent!
- but 2S-2x11B7 is far too small - perhaps this clone has a frameshift mutation close to CBD96?
- or maybe it has no insert after all

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